Differential Contribution of Reticulospinal Cells to the Control of Locomotion Induced By the Mesencephalic Locomotor Region

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Submitted 23 April 2003; accepted in final form 30 April 2003

Brocard, Frédéric and Réjean Dubuc. Differential contribution of reticulospinal cells to the control of locomotion induced by the mesencephalic locomotor region. J Neurophysiol 90: 1714–1727, 2003. First published May 7, 2003; 10.1152/jn.00202.2003. In lampreys as in other vertebrates, the reticulospinal (RS) system relays inputs from the mesencephalic locomotor region (MLR) to the spinal locomotor networks. Semi-intact preparations of larval sea lamprey were used to determine the relative contribution of the middle (MRRN) and the posterior (PRRN) rhombencephalic reticular nuclei to swimming controlled by the MLR. Intracellular recordings were performed to examine the inputs from the MLR to RS neurons. Stimulation of the MLR elicited monosynaptic excitatory responses of a higher magnitude in the MRRN than in the PRRN. This differential effect was not attributed to intrinsic properties of RS neurons. Paired recordings showed that at threshold intensity for swimming, spiking activity was primarily elicited in RS cells of the MRRN. Interestingly, cells of the PRRN began to discharge at higher stimulation intensities only when MRRN cells had reached their maximal discharge rate. Glutamate antagonists were ejected in either nucleus to reduce their activity. Ejections over the MRRN increased the stimulation threshold for evoking locomotion and resulted in a marked decrease in the swimming frequency and the strength of the muscle contractions. Ejections over the PRRN decreased the frequency of swimming. This study provides support for the concept that RS cells show a specific recruitment pattern during MLR-induced locomotion. RS cells in the MRRN are primarily involved in initiation and maintenance of low-intensity swimming. At higher frequency locomotor rhythm, RS cells in both the MRRN and the PRRN are recruited.

INTRODUCTION

Locomotion is a rhythmic motor activity generated by spinal neural networks, referred to as a central pattern generator (CPG) (Grillner and Wallén 1999; Rossignol and Dubuc 1994). These spinal networks are activated, modulated, and stopped by supraspinal structures required for initiation and adaptive control of goal-directed locomotion (Grillner et al. 1997; Mori et al. 2001). One such structure is the “mesencephalic locomotor region” (MLR), first described in cats by Shik et al. (1966). It controls the intensity of locomotion in a “nearly linear” fashion, such that progressively higher stimulation intensities lead to a higher frequency rhythm. Most of the connectivity to and from the MLR has been studied in mammals, and the major findings have been reviewed (Grillner et al. 1997; Jordan 1998). There are several lines of evidence showing that stimulation of the MLR activates the CPG through a monosynaptic activation of reticulospinal (RS) neurons located in both thepons and the medulla. In cats, it appears that the medullary reticular formation and descending pathways through the ventrolateral funiculus play a predominant role in MLR-induced locomotion (Garcia-Rill and Skinner 1987a,b; Shefchyk et al. 1984; Steeves and Jordan 1980). Despite the numerous studies in higher vertebrates, our knowledge of the cellular mechanisms by which the MLR activates RS cells and the specific role of different reticular nuclei in the MLR-induced locomotion has remained parsimonious, partly due to the complexity of the mammalian nervous system.

The MLR has been described in lampreys, a lower vertebrate model with a comparatively simpler nervous system (Sirota et al. 2000). This locomotor region provides mixed monosynaptic inputs, mostly glutamatergic and cholinergic, onto RS cells (Le Ray et al. 2003). The RS system of lampreys constitutes the main descending pathway and provides excitatory inputs to the spinal locomotor networks (Buchanan et al. 1987; Ohta and Grillner 1989). Based on histological criteria, the reticular formation has been divided into four nuclei (Nieuwenhuys 1972, 1977): the mesencephalic reticular nucleus (MRN) and three rhombencephalic reticular nuclei, the anterior (ARRN), the middle (MRRN), and the posterior (PRRN). The MRN and PRRN are the primary sources of inputs to the spinal cord, representing ~90% of the descending neurons (Bussières 1994). Electrophysiological studies revealed that RS cells display a variety of influences on the spinal motor output (Wannier et al. 1998; Zelenin et al. 2001). Despite the overlapping motor effects between different subdivisions of the RS system, the MRRN was suggested to play a predominant role in the control of equilibrium and steering during locomotion (Delagina et al. 1993; Fagerstedt et al. 2001; Ullén et al. 1996), whereas the PRRN was proposed to contribute to the initiation of locomotion (McClellan 1988; Ohta and Grillner 1989).

In the present study, we have used a semi-intact lamprey preparation to investigate the connectivity between the MLR and RS neurons. We characterized the synaptic inputs from the MLR in different groups of RS cells and determined the recruitment pattern of RS cells during MLR-induced locomotion. Subsequently, we investigated the respective contribution...
of the MRRN and the PRRN on swimming induced by MLR stimulation. To do this, the glutamate antagonists, 6-cyano-7-nitroquinoxaline-2,3-dione and 2-amino-5-phosphonovaleric acid, were ejected in either nucleus to reduce their activity. Preliminary results have been reported in abstract form (Brocard and Dubuc 2002).

METHODS

Semientire preparation

Experiments were performed on 42 larval (9–14 cm) sea lampreys, Petromyzon marinus. All surgical and experimental procedures conformed to guidelines of the Canadian Institutes for Health Research and were approved by the University Animal Care and Use Committee. The animals were anesthetized with tricaine methanesulphonate (MS 222, 100 mg/l), incised along the ventral midline and eviscerated. The dissection and experiments were performed in cold oxygenated Ringer (8–10°C) with the following composition (in mM): 130 NaCl, 2.1 KCl, 2.6 CaCl₂, 1.8 MgCl₂, 4 HEPES, 4 dextrose, and 1 NaHCO₃, at pH 7.4. The animals were pinned down onto silicone elastomer (Sylgard), and a dorsal incision was used to expose the rostral spinal cord and the brain, whereas the tail was left intact. A decerebration was performed by cutting and removing the brain tissue rostral to the mesencephalon. The semi-intact preparation was then transferred to an experimental chamber continually perfused with Ringer at a rate of ~4 ml/min. The preparation was mounted by pinning the cut edges of the rostral notochord and the cranium while the tail was free to move. An incomplete petroleum jelly (Vaseline) barrier was built at the rostral notochord and the cranium while the tail was free to move. /H11011

Methodological considerations

Intracellular recordings were made from RS neurons in the MRRN and PRRN using sharp glass microelectrodes filled with 4 M potassium acetate (80–130 MΩ). Recordings were made using an Axoclamp 2A amplifier (Axon Instruments, CA; sampling rate: 2–10 kHz) in bridge or discontinuous current-clamp modes. Most of the RS cells that were recorded in the MRRN or PRRN were large enough to be visualized under the dissecting microscope (see Rovainen 1967). They were thus visually impaled and not systematically identified by an antidromic stimulation of the spinal cord. On the other hand, the RS cells of the PRRN that were blindly impaled were identified by antidromic stimulation. Only RS neurons with a stable membrane potential (≥15 min after impalement) more hyperpolarized than ~70 mV were included in the study. The input resistance of RS cells was measured by injecting hyperpolarizing current pulses (0.25–0.5 nA, 500 ms). The rheobase was defined as the minimum current intensity necessary to fire the cell.

Single stimuli of increasing intensity were delivered every 30 s to examine the synaptic inputs from the MLR to RS cells. At least three traces were averaged at each stimulus intensity to improve the signal-to-noise ratio. The stimulus/response relationships were then plotted and fitted by a sigmoidal function using Origin 7.0 software (OriginLab, Northampton, MA). The peak amplitude of the evoked postsynaptic potentials was measured as the largest voltage deflection from the resting membrane potential, whereas their durations were measured at 50% of peak amplitude. The latency was measured from the stimulus artifact to the onset of the response. The slope was estimated by measuring the greatest rising phase between two consecutive points on the evoked postsynaptic potentials. The time to peak was the duration from the onset to the peak amplitude of the responses.

Locomotion was induced by repeated electrical stimulation (1.5-ms square-wave pulses, 2 Hz, 0.5–15 μA) of the MLR with a glass-coated tungsten microelectrode. Details of the systematic search of the MLR have been published elsewhere (Sirota et al. 2000). The trains of stimulation lasted 40 s. Intervals between successive trials were not <3 min. The stimulus artifacts on intracellular recordings were clipped using a homemade software. Locomotion was monitored by electromyographic (EMG) recordings using two pairs of Teflon-coated stainless steel wires (50 μm diameter; California Fine Wire, Grover Beach, CA) inserted into the myotomes between segmental levels 20 and 25. The EMG signals were amplified (×1,000), filtered (bandwidth: 30 Hz to 1 kHz) and recorded with a sampling rate of 5 kHz. The amplitude, the duration, and the frequency of the EMG bursts were measured by using a homemade software peak detector and level detector. The cycle duration (swimming frequency) was measured as the interval between the onset of two consecutive bursts on the same side. The measurements were made on EMG signals previously rectified and filtered (time constant: 10 ms). For each testing session, the parameters were calculated by averaging ~10 consecutive cycles during a steady-state sequence of locomotion. The semi-intact preparation allows us to visualize directly the locomotor movements, thus bilateral EMG recordings were not systematically illustrated in the figures (see, however, Fig. 7).

Drugs application

To reduce excitation in the MRRN or the PRRN, the two glutamate receptor antagonists, 2-amino-5-phosphonovaleric acid (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) were pressure ejected over the entire RS nucleus on both sides. The two drugs were obtained from Sigma Chemical (Oakville, Ontario). They were diluted in Ringer solution adjusted to pH 7.4 and pressure-applied locally onto RS neurons through a glass micropipette using a Picospritzer (General Valve, Fairfield, NJ). The inactive dye Fast Green was added to the drug solution to monitor the size and exact location of the ejections, and the green stain made over the brain tissue was redrawn on a schematized brain stem. To prevent spreading of the drugs within the brain stem, the outflow apparatus was placed near the ejection site. Moreover, to confirm a local action of the drugs, the activity of RS cells located in the adjacent noninactivated reticular nucleus was recorded. When the ejections were made over the MRRN, the activity of RS cells located in the rostral part of the PRRN was recorded, whereas the ejections were made over the PRRN, caudal RS cells of the MRRN were recorded. Trials that exhibited depression of synaptic transmission in the adjacent noninactivated nucleus were not considered. After each application, a washout period of varying duration (several minutes to >1 h), was allowed for recovery.

Data analysis

Data acquisition was carried out on an IBM computer via a Digitador 1322A interface and using Clampex 8 software (Axon Instruments). Off-line analysis was performed with Clampfit 8.0 software. Data in the text and figures are given as means ± SE. All statistical analyses were carried out using Origin software (OriginLab). A Student’s t-test was used when means of two groups were compared, whereas a one-way ANOVA was used for multiple group comparisons. In the latter case, a Tukey’s test was used for post hoc analysis. Differences were considered statistically significant when P < 0.05.

RESULTS

Differential effects of the MLR onto reticulospinal cells

The connectivity between the MLR and RS cells was first investigated by applying single stimulation shocks in the MLR. Intracellular recordings were made from 70 RS cells in 15 preparations. Of these, 42 cells were located in the MRRN and 28 in the PRRN. The evoked postsynaptic potentials (PSPs)
were examined and compared with respect to their peak amplitude, duration, and slope.

To study comparable responses in RS cells of the MRRN and PRRN, we first determined the MLR stimulation intensity needed to evoke maximal subthreshold responses in those two reticular nuclei. The synaptic properties of RS neurons were assessed for their stimulus/response relationship. Interestingly, we found that within a preparation, the threshold intensity (T) for evoking depolarizing responses in both nuclei was identical. In all cells, increasing the stimulus intensity produced synaptic responses with increasing amplitudes, but with a latency remaining nearly constant (Fig. 1A). After normalization with respect to the maximal amplitude, the stimulus/response relationships were similar for MRRN and PRRN cells (P = 0.99; Fig. 1B). The amplitude of the PSPs increased rapidly with the stimulation intensity, up to 8T, to eventually plateau at higher stimulation intensities. In some cases, higher stimulation intensities produced action potentials, but these responses were not considered for analysis. In the fast rising part of the curves, the stimulus intensity required to achieve 20, 40, 60, and 80% maximal PSP amplitude did not differ significantly in RS cells of the two nuclei (P = 0.88, 0.85, 0.28, and 0.26 for 20, 40, 60, and 80%, respectively; Fig. 1B).

RS cells were recorded from four zones of the reticular formation, arbitrarily defined as zones 1 and 2 corresponding to the rostral and caudal halves of the MRRN and zones 3 and 4, corresponding to the rostral and caudal halves of the PRRN, respectively (Fig. 2A1). To compare the synaptic responses between cells in the two nuclei, the MLR was stimulated >8T. Representative postsynaptic responses from RS cells in the four different zones of the reticular formation are illustrated in Fig. 2A2. The size of the maximal PSPs showed a gradual decrease along the rostrocaudal axis of the reticular formation (P < 0.001; Fig. 2B1), whereas the response duration increased significantly (P < 0.001; Fig. 2B2). The time to reach peak amplitude was less in the rostral cells of the MRRN than in PRRN cells (P < 0.001; Fig. 2B3). The former also displayed a higher slope (P < 0.001; mean latency: 5.1 ± 0.3 and 8.9 ± 0.5 ms for RS cells of the MRRN and PRRN, respectively; see Figs. 1A and 2A2).

**Synaptic connectivity from the MLR to RS cells**

Experiments were carried out to determine whether monosynaptic or polysynaptic connections were present between the MLR and RS neurons. Maximal subthreshold responses were recorded in RS cells of the MRRN (n = 3) and PRRN (n = 3) under control conditions and in the presence of a high concentration of divalent cations (10.8 mM Ca^{2+}/7.2 mM Mg^{2+}) in the bath to abolish polysynaptic pathways. In the high-divalent cation solution, the amplitude of PSPs evoked by stimulation of the MLR was reduced by 16 ± 13% in cells of the MRRN and the PRRN (Fig. 3A, I and 2, P < 0.05, n = 6). The response that remained in RS cells of either nucleus exhibited a constant latency during high-frequency stimulation at 25 Hz (Fig. 3B, I and 2). There was often a late excitatory component in the synaptic response of PRRN cells. Both early and late components persisted (Fig. 3B2). These results provide good evidence that a large part of the excitation from the MLR to RS cells of the MRRN and PRRN results from a monosynaptic connection. To validate this method as a test for monosynaptic connections in lampreys, we stimulated the trigeminal nerve, which elicits disynaptic excitation in RS neurons (Viana Di Prisco et al. 1995), and the synaptic responses were abolished by the high concentration of divalent cations (n = 3; not illustrated).

We examined whether the decrease of MLR inputs by high-divalent cations resulted from either a disappearance of polysynaptic pathways or a reduction of monosynaptic transmission. Indeed, the magnitude of monosynaptic inputs from large RS cells onto giant interneurons in the lamprey spinal cord was shown to be decreased in high concentrations of Mg^{2+} ions (Rovainen 1967), which antagonize Ca^{2+} and reduce transmitter release. Therefore we studied the time course of the synaptic transmission between MLR and RS cells during perfusion of Ca^{2+}-free solution replacing normal Ringer. Composite PSPs in RS cells of the MRRN and PRRN showed a gradual decrease in amplitude (Fig. 3C, I and 2). The area of PSPs evoked in the MRRN (n = 3) and PRRN (n = 3) showed a gradual reduction without failure or any abrupt decrease (Fig. 3D, I and 2). Taken together, these results suggest that most, if not all of the synaptic input from the MLR to RS cells is monosynaptic.
Electrophysiological properties of RS cells

The amplitude of PSPs may have been influenced by some of the intrinsic properties of the postsynaptic cell. To test this, a total of 106 RS cells from the four predefined zones were recorded and their electrophysiological properties compared. No difference was observed in their resting potential (Fig. 4A). RS cells in the four zones exhibited a similar action potential threshold, which was on average 21.3, 19.6, 21.5, and 22 mV less negative than the resting membrane potential of neurons in zones 1–4, respectively (Fig. 4A). There were no significant differences in the membrane resistance nor the rheobase across the four zones. On the other hand, RS cells in the MRRN (zones 1–2) tended to have a lower membrane resistance (Fig. 3B) and a higher rheobase (Fig. 3C), suggesting that they would be less excitable to synaptic inputs than PRRN cells (zones 3 and 4). Yet MRRN cells displayed the largest synaptic responses to MLR stimulation (see preceding text), suggesting that the intrinsic properties we have measured in RS cells were not responsible for the differential effect of the MLR in different RS cells.

RS cells in the MRRN and PRRN display a different recruitment pattern

To further investigate the consequence of the differential inputs from the MLR to RS neurons in the control of locomotion, the recruitment pattern of RS cells was examined during MLR-induced locomotion. Paired intracellular recordings were carried out from RS cells in the MRRN and PRRN (3 pairs in 3 different preparations). The MLR was stimulated for 40 s at increasing currents with constant frequency (2 Hz). The average threshold for inducing swimming was systematically higher than the threshold for evoking single PSPs in RS cells.

FIG. 2. Differential effect of the MLR on RS cells of the MRRN and PRRN. A1: schematic representation of the experimental paradigm. The 2 reticular nuclei were arbitrary subdivided in 2 equal areas: zone 1 and 2 for the MRRN, zone 3 and 4 for the PRRN. A2: representative maximal response evoked by the MLR in RS cells located in the 4 different zones. All traces are averages of 5 sweeps. B: histograms depicting the mean peak amplitude (B1), the duration (B2), the time to peak (B3), and the slope (B4) of maximal subthreshold responses in RS neurons located in the 4 zones. Statistical significance: *P < 0.05, **P < 0.01, ***P < 0.001 (1-way ANOVA followed by a Tukey’s post hoc analysis).
FIG. 3. Monosynaptic inputs from the MLR to RS cells of the MRRN and PRRN. A: MLR-evoked PSPs in RS neurons of the MRRN (A1) and PRRN (A2) recorded under control conditions and in the presence of 10.8 mM Ca$^{2+}$/7.2 mM Mg$^{2+}$ (40-min exposure). All traces are averages of 5 sweeps. B: the remaining response in high-divalent cation solution persisted in MRRN (B1) and PRRN (B2) cells during high-frequency stimulation (25 Hz) and occurred with constant latency. Note that both components of the response in RS cells of the PRRN persisted (see arrows). C: effect of Ca$^{2+}$-free physiological solution on PSPs produced by stimulation of the MLR. A typical gradual reduction of the PSPs is illustrated for 1 MRRN (C1) and 1 PRRN (C2) neuron after 10-, 15-, 20-, 25-, and 30-min exposure. All traces are averages of 5 sweeps. D: time course (relative to control value) of the PSP area in representative MRRN (D1) and PRRN (D2) cells during exposure to the Ca$^{2+}$-free solution.
(3.2 ± 0.7 vs. 1.5 ± 0.2 μA, *P* < 0.05). At threshold intensity for swimming, spikes were primarily elicited in MRRN cells, whereas PRRN neurons displayed only subthreshold depolarizations (Fig. 5A). PRRN cells began to discharge at higher stimulation intensities of the MLR (Fig. 5B). The cells displayed membrane oscillations in phase with the EMG activity (see expanded records in Fig. 5).

For all experiments, the cycle duration decreased in a nearly linear fashion with the stimulation intensity applied to the MLR (*P* < 0.001, *r* = -0.95, Fig. 6A1) (see also Sirota et al. 2000). As the stimulation intensity was increased, the firing frequency of MRRN cells increased linearly to eventually reach a plateau at 2.5–3T (Fig. 6A1, *P* < 0.001; 1-way ANOVA). Interestingly, the PRRN cells began to be recruited only when MRRN cell discharge had reached a plateau. The discharge rate of PRRN cells increased linearly with the stimulation intensity. For a given stimulation intensity, PRRN cells exhibited a significantly lower firing frequency than cells of the MRRN (*P* < 0.001). Cumulative data from three pairs of cells in three different preparations showed a similar recruitment pattern (Fig. 6A2). At low stimulation intensity, RS cells of the MRRN tended to show a slow build-up of discharge as compared with higher intensities (Fig. 6B, compare also top traces in Fig. 5, A and B). With an increase in stimulation intensity, the delay between the beginning of stimulation and that of spiking in the RS cells decreased (*P* < 0.05; Fig. 6C1) and the discharge of the cells over-lasted the stimulation period (*P* < 0.05; Fig. 6C2, compare also top in Fig. 5, A and B).

**Effects of glutamate antagonists ejected over the MRRN on MLR-induced locomotion**

Because the MLR input to RS cells is in large part glutamatergic (Le Ray et al. 2003), the two glutamatergic antagonists, CNQX (1.25 mM) and AP5 (5 mM), were pressure ejected over the entire MRRN on both sides to study contribution of this nucleus to locomotor activity (Fig. 7A). Under this condition, the magnitude of the synaptic responses elicited in RS cells of the MRRN by stimulation of the MLR was dramatically decreased (Fig. 7B). The remaining excitation was likely to be cholinergic as we have previously shown by bath applying the glutamate receptors antagonists (Le Ray et al. 2003). We then investigated the effects of the drugs ejections on ongoing locomotor activity induced by MLR stimulation. Within seconds after the ejection, spiking activity decreased in the recorded cell as well as the underlying depolarization, which rapidly became subthreshold (Figs. 7 C and D, 1–3). Both the frequency of swimming and the amplitude of EMG bursts decreased even when the MLR stimulation was maintained (*n* = 2).

The glutamate antagonists were ejected prior to MLR stimulation in eight preparations. In six of these, swimming activity could not be elicited at low stimulation intensities (compare Fig. 8, A1 with B1) and, the threshold intensity for inducing locomotion was nearly doubled (Fig. 8C; *P* < 0.05). The cycle duration of locomotion increased significantly by 39–71% depending on the stimulation intensity (Fig. 8D, *n* = 3, *P* < 0.001). Low-frequency swimming emerged with on average 78 ± 3.5% lower burst amplitude (Fig. 8B2). The decrease in burst amplitude was statistically significant (*P* < 0.05; paired t-test) although it was not accompanied by a significant change in burst duration. Membrane potential oscillations and spiking activity were abolished in cells of the PRRN even at high MLR stimulation intensities (compare Fig. 8, A2 and B2). On the other hand, the subthreshold PSPs elicited by MLR stimulation were not affected (Fig. 8B2, inset).

**Effects of glutamate antagonists ejected over the PRRN on MLR-induced locomotion**

Pressure-ejection of glutamatergic antagonists over the PRRN on both sides, failed to block locomotion even at low
stimulation intensity \((n = 7\) preparations). The threshold for swimming was not changed (Fig. 9C), but there was a significant increase in the cycle duration \((14.9\%, P < 0.01,\) Fig. 9D). This increase could occur at stimulation intensities below those needed to induce action potentials in the recorded RS cells of the PRRN (Fig. 9D and compare A3 with B3). Indeed, the increase of the cycle duration began at 1.5T, i.e., just before the recruitment of discharges in RS cells in the PRRN. On the other hand, the firing frequency of the MRRN cell was not affected \((P = 0.82,\) compare Fig. 9, A1 with B1). Although there was an increase of the cycle duration, the burst amplitudes and durations were not significantly altered.

Finally, because the ejections over the MRRN or the PRRN depressed MLR-induced swimming, microejections were made
over both reticular nuclei. Under these conditions, swimming was completely blocked, even at high stimulation intensities (not illustrated).

**DISCUSSION**

In this report, a semi-intact lamprey preparation was used to characterize the synaptic inputs from the MLR to RS cells in the two main reticular nuclei of the rhombencephalon. We provide the first functional evidence that the control of swimming is exerted, at least partly, by a differential effect of the MLR onto the reticular formation. The MLR elicited monosynaptic EPSPs of a greater magnitude in RS cells of the MRRN than of the PRRN. As the MLR stimulation intensity was increased, RS cells of the MRRN were first recruited until they reached a plateau in their firing rate. Only then, RS neurons of the PRRN were recruited. Local application of glutamate antagonists in the MRRN increased the MLR stimulation threshold needed to evoke locomotion and depressed the locomotor activity. Ejections over the PRRN did not block locomotion but slightly decreased the swimming frequency. Collectively, our results suggest that the relative contribution of the MRRN and the PRRN in MLR-induced locomotion is different. MRRN cells seem primarily involved in initiation and maintenance of low swimming frequency, whereas both MRRN and PRRN cells are recruited for higher frequency locomotion.

**Synaptic inputs from the MLR to RS cells**

By testing the synaptic connections, we showed that the largest part (if not all) of the MLR inputs onto RS cells is monosynaptic. Reticulospinal cells of the PRRN showed synaptic responses with a longer latency than those of the MRRN (ca. 3.8 ms). Although we cannot exclude the presence of an additional relay neurons between the MLR and PRRN cells, this difference is likely to result from the longer distance between the MLR and the PRRN compared with the MRRN.

In addition to glutamatergic inputs, we have recently shown that the MLR exerts a cholinergic influence onto rhombencephalic RS cells in lampreys (Le Ray et al. 2003). A nicotinic antagonist depressed the MLR-evoked responses in RS cells and increased the threshold intensity for inducing swimming in a semi-intact preparation. If the cholinergic inputs play a role

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![Graphs](http://jn.physiology.org/))
in the control of locomotion, the present study demonstrates that in absence of glutamatergic transmission, the cholinergic inputs that originate from the MLR are not sufficient to induce locomotion. Indeed, glutamate antagonists ejected over the two reticular nuclei completely blocked MLR-induced locomotor activity. This suggests that cooperation with glutamatergic inputs would likely be needed for the cholinergic inputs to induce locomotion. This could be an important feature of the MLR command to RS cells in lampreys.

The stimulus/response relationships were similar in RS cells of the MRRN and PRRN. The stimulation threshold to evoke synaptic responses was also the same. These observations were consistent either with an activation of MLR cell(s) sending en passant collaterals to multiple levels of the reticular formation or

FIG. 7. Effects of blocking glutamatergic inputs into the MRRN on ongoing swimming activity induced by MLR stimulation. A: schematic representation of the experimental paradigm depicting the area of the MRRN microinjected by the glutamatergic antagonists. B: the MLR-evoked PSPs in 1 RS cell of the MRRN are reduced but not completely blocked by a local ejection of 6-cyano-7-nitroquinolxalene-2,3-dione (CNQX) and 2-amino-5-phosphonovaleric acid (AP5). C: intracellular recording (top) from a RS cell in the MRRN during swimming evoked in a semi-intact preparation by MLR stimulation at a low intensity (2T). Repeated local ejections (†) of CNQX (1.25 mM) and AP5 (5 mM) were made onto the MRRN covering the entire nucleus on both sides. The EMGs (bottom) were recorded at segmental level 20 ipsi- (iEMG) and contralateral (cEMG) to the intracellular recordings. The horizontal bar below the recordings indicates the duration of the MLR stimulation. Parts of the recording indicated by shaded areas in C are shown at a faster time scale in D, 1–3.
FIG. 8. Effects of blocking glutamatergic inputs into the MRRN on the initiation of locomotion by the MLR. A and B: intracellular recording of a typical PRRN neuron (top) and EMG (bottom) activities for low (1.5T, left) and high (3T, right) MLR stimulation intensities, before (A) and after (B) ejections of glutamatergic antagonists (CNQX and AP5) over the MRRN. The size of the ejection is illustrated on a schematized brain stem in B, 1 and 2. The EMG activity was recorded at segmental level 20 ipsilateral to the intracellular recordings. B2, inset, illustrates averages of subthreshold responses evoked in the PRRN cell by single pulse stimulation of the MLR (3T) before and after ejections of glutamate antagonists over the MRRN. C: histogram depicting the MLR stimulation threshold for swimming before and after ejections of the glutamate antagonists over the MRRN. The threshold values were expressed in percentage of the control value. D: relationship between cycle duration of swimming movements and stimulus intensity of the MLR before (black line) and after (gray line) pressure ejection of CNQX and AP5 over the MRRN. T represents the threshold intensity needed to induce locomotion. Statistical significance: *P < 0.05, ***P < 0.001.
with a simultaneous recruitment of two intermingled populations of MLR-projecting cells, one terminating in MRRN and the other in PRRN. According to the latter hypothesis, the cells would be uniformly spread over in the same area because the stimulation intensities to reach a maximum in MRRN and PRRN cells were also similar. It is thus unlikely that increasing the stimulation intensity would recruit two spatially distinct populations of MLR neurons.

A salient finding of the present study is that the monosynaptic MLR-evoked responses had a significantly greater magnitude in RS cells of the MRRN as compared with those of the PRRN. A similar difference in the strength of synaptic inputs between MRRN and PRRN RS cells was previously shown for sensory-evoked motor responses (Fagerstedt et al. 2001; Viana Di Prisco et al. 2000). The present study demonstrates that some of the intrinsic properties of RS cells do not appear to be responsible for this difference. Their membrane excitability increases from rostral to caudal parts of the rhombencephalon. This would result in synaptic responses of greater magnitude in PRRN cells for a similar input, which is not what we have observed in the present study. Other factors that might contribute are: a relatively less extensive MLR projection to PRRN neurons or differences in postsynaptic receptors (e.g., density, affinity, postsynaptic placement or even receptor-mediated currents). The PSPs induced in PRRN neurons displayed a longer

![Figure 9](image-url)

**FIG. 9.** Effects of blocking glutamatergic inputs into the PRRN on MLR-induced swimming. A, 1 and 2, illustrates typical intracellular recordings of 1 MRRN cell and EMG activities during swimming induced by stimulation of the MLR at 3T. B, 1 and 2, is the same as A, 1 and 2, but after ejection of CNQX and AP5 over the PRRN. The size of the ejection is illustrated on a schematized brain stem in B1. Shaded areas are shown at a faster time scale in A3 and B3. C: histogram depicting the MLR stimulation threshold for swimming before and after ejections of the glutamate antagonists over the PRRN. The threshold values were expressed in percentage of the control value. D: relationship between cycle duration of swimming movements and stimulus intensity of the MLR before (black line) and after (gray line) pressure ejection of CNQX and AP5 over the PRRN. T represents the threshold intensity needed to induce locomotion. Statistical significances: *P < 0.05, **P < 0.01, ***P < 0.001.
time to peak and a slower rise and had a longer duration. This could be due to a nonsynchronous presynaptic discharge or to ionic currents with a slower kinetic in PRRN cells, such as the ones mediated by N-methyl-D-aspartate receptors. On the other hand, fast AMPA/kainate or nicotinic currents could predominate in RS cells of the MRRN. Other experiments are needed to test these hypotheses.

Role of the two reticular nuclei in MLR-induced swimming

A considerable body of evidence suggests an involvement of the RS system in initiating and modulating locomotor activity in all vertebrates (Drew 1991; Jordan 1998; Ohta and Grillner 1989; Orlovsky 1972). As was first proposed in cats by Orlovsky (1970), RS cells play a key role in relaying the MLR command to the spinal CPGs. This was demonstrated in a number of vertebrate species afterwards (see for review Grillner et al. 1997). Our experiments in lampreys concur with this because glutamate antagonists pressure ejected over both MRRN and PRRN prevented MLR-induced swimming.

The MRRN plays a prominent role in the initiation as well as in the maintenance of MLR-induced locomotion. Ejections of glutamate antagonists over the MRRN were accompanied by a reversible increase of the stimulation threshold needed to elicit locomotion and the suppression of ongoing swimming, induced by a mild stimulation of the MLR. These results are consistent with the discharge pattern of large MRRN cells (Müller cells) being correlated with locomotor activity. Their discharge started prior or at the onset of swimming. This finding is highly indicative that Müller cells may participate in initiation of locomotion. However, we cannot conclude that they are essential because they represent <5% of the ~300 cells of the MRRN (Bussières 1994). Rovainen (1967) showed that a high-frequency stimulation of a single Müller cell does not generate swimming. Furthermore, lesion studies indicate that Müller cells are not required for escape swimming (McClellan 1988). However, the functional organization of RS system in “sensory-evoked locomotion” and “goal-directed locomotion” could be different. As demonstrated in zebrafish larva (Gahtan et al. 2002) and lamprey (Viana Di Prisco et al. 1997), in the context of an escape-behavior, the RS system is widely activated, suggesting that in addition to Müller cells, smaller RS neurons could effectively contribute to the generation of escape-swimming. After local application of glutamate antagonists over the MRRN, the activity of PRIN cells was depressed during swimming. This could result from a weaker efferent feedback from the CPG on the PRIN due to a reduced swimming activity. The disappearance of a potential interaction between the MRRN and the PRIN could also be involved. However, in addition to our paired recordings, dual intracellular recordings were made in a previous electrophysiological study (Dubuc et al. 1993), and no interactions were found between RS cells of the MRRN and those of the PRIN.

Blockade of glutamatergic transmission in the PRIN did not prevent swimming but decreased its frequency, suggesting that this nucleus is not essential to initiate locomotion but may modulate the locomotor rhythm. This is consistent with the previously observed acceleration of ongoing swimming evoked by PRIN stimulation (Wannier et al. 1998). When stimulating the MLR near threshold, swimming decelerated after the PRIN blockade. The MLR stimulation was then subthreshold for recruiting the PRIN cells that were recorded in the present study, our intracellular recordings being likely biased toward the larger cells in the nucleus. It is thus likely that the deceleration of swimming was due to effects of the glutamate antagonists on other RS cells in the PRIN, perhaps smaller cells that were not recorded. Bussières (1994) reported that >75% of the ~700 RS cells in the PRIN have a diameter of <20 μm.

Based on these considerations, we conclude that MLR inputs are not evenly distributed in the reticular formation: large RS cells of the MRRN are preferentially recruited at low MLR stimulation intensities whereas those of the PRIN are recruited at higher intensities.

Recruitment pattern of RS cells and its functional implication

As the MLR stimulation intensity is increased, the firing rate of MRRN cells increases to eventually saturate. Interestingly, it was only then that PRIN cells began to be recruited and underwent an increase in their firing rates. However, for a given stimulation intensity, RS cells of the PRIN were found to systematically generate higher firing frequencies than those of the PRIN. This could result from a weaker excitatory drive from the MLR to RS cells of the PRIN. On the other hand, Rouse et al. (1998) showed that the proportion of RS cells exhibiting tonic firing to a sustained depolarizing current was higher in MRRN than in the PRIN. A larger Na⁺ or Ca²⁺ influx in RS cells of the MRRN could be an appealing explanation for the higher frequency of discharge in those cells.

RS cells have been found to excite spinal neurons through glutamatergic transmission (Buchanan et al. 1987; Ohta and Grillner 1989), and higher locomotor frequencies can be achieved by increasing the excitatory drive to the CPG (Brodin et al. 1985; Trávén et al. 1993). We now describe that RS cells are progressively recruited by the MLR, and this would fit well with a nearly linear control of the swimming frequency exerted by the MLR.

Comparative aspects

The MLR is a functionally defined brain structure highly conserved in all vertebrate classes (Grillner et al. 1997). Several lines of evidence suggest that cholinergic neurons may be part of the MLR in mammals (Garcia-Rill 1991). Immunohistochemical data indicate that there are also cholinergic cells in the MLR of lampreys (Le Ray et al. 2003; Pombal et al. 2001). Furthermore, we have recently demonstrated that cholinergic inputs onto RS cells, play a role in the initiation of swimming (Le Ray et al. 2003). Our present results emphasize a primary role of glutamatergic transmission onto RS cells in the control of locomotion. Similar evidence has also demonstrated in cats (Noga et al. 1988).

In mammals, neurophysiological investigations have demonstrated that the medial medullary reticular formation plays an important role in goal-directed behavior by relaying MLR inputs to the spinal locomotor networks via the ventrolateral funiculus (Atsuta et al. 1990; Garcia-Rill and Skinner 1987a,b; Iwakiri et al. 1995; Marlinsky and Voitenko 1991; Noga et al. 1991; Orlovsky 1970). These findings are supported by neuroanatomical evidence showing dense projections to the med-
ullary reticular formation from MLR, specifically to the n. gigantocellularis and n. magnocellularis (Lai et al. 1999; Skinner et al. 1990; Steeves and Jordan 1984). We show here, in lampreys, that MLR inputs are larger in RS cells of the MRRN than those of the PRRN. The phylogenetic homology between the reticular formation of mammals and that of the lamprey is not established, but according to its anatomical position, the MRRN would correspond to the pontine reticular formation of mammals, whereas the PRRN would be part of the medullary reticular formation. Large RS cells in the MRRN (Müller cells) send their axons medially in the spinal cord (Rovainen et al. 1973). In cats, the ventromedial pathways do not seem to be as important as the ventrolateral pathways to induce locomotion by the MLR (Steeves and Jordan 1980). By examining the recruitment pattern of RS cells as well as blocking their glutamatergic excitatory inputs, we found that cells of the MRRN seem to contribute significantly to the initiation of locomotion in lampreys. The possible homology between this reticular nucleus and the pontine reticular formation in mammals would indicate that the role of the latter in locomotion might have been underestimated. In accordance with this, it was recently shown in cats and rats that the pedunculopontine nucleus, one of the major components of the MLR, has strong excitatory effects on the caudal pontine reticular formation (Garcia-Rill et al. 2001; Homma et al. 2002). Moreover, pontine RS neurons with fast-conducting axons exhibit rhythmic discharges during locomotion in cats (Prentice and Drew 2001; Shimamura et al. 1985). MLR-induced locomotion is abolished after a bilateral lesion of the pontine reticular formation (Shimamura et al. 1984). Altogether, these observations indicate that pontine RS cells play a role in the initiation of locomotion in mammals as we are now showing in lampreys.

The authors express their gratitude to D. Veilleux for assistance with the experiments and to C. Vailiquette for expertise in computer programming. We are grateful to F. Auclair and K. Fénelon for critical review of this manuscript. We thank W. D. Swink and M. K. Jones from the Lake Huron Biological Station as well as J. E. Gersmehl, E. Howe, and W. Boufard from the U.S. Fish and Wildlife Service for the kind supply of lampreys.

DISCLOSURES

This work was supported by the Canadian Institutes for Health Research (CIHR), The Canadian Neurotrauma Research Program and the Fonds pour la Formation des Chercheurs et l’Aide à la Recherche (Québec). F. Brocard received a fellowship from the Fondation pour la Recherche Médicale (France) and the Jasper Fellowship program (Québec).

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